METHODS

Cells and viruses. SARS-CoV (Tor3 strain) was inoculated onto Vero E6 cells grown in Dulbecco's modified eagle medium supplemented with 10% foetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.22 mg/ml l-glutamine at 37^{o}C with 5% CO_2. Cell supernatant was harvested (300ml) with a SARS-CoV $TCID_{50}$ titre of $5x10^7$/ml and virus was pelleted using a Beckman SW32 rotor at 28000 rpm for 90 minutes. The virus pellet was resuspended in PBS buffer, layered onto of a 12-30% iodoxanol gradient (Optiprep) and centrifuged in a SW40 rotor at 38000 rpm for 2.5 h. Fractions (0.7 ml) were collected from the gradient with an Auto Densi-Flow gradient collector (Labcono) and dialysed against PBS. SARS-CoV-enriched fractions were checked by SDS-Page and Western blotting, and rendered non-infectious by irradiation in a gamma cell on dry ice with a 2 Mrad exposure for 90 minutes. The dose was chosen as sufficient for viral inactivation[1], while retaining antibody[1,2] or enzyme functional activities[3]. Previous studies have indicated that viral RNA is highly sensitive to radiation and that viruses were by 2 Mrad[1], even when cooled to minimize heat damage to proteins, whereas antigenicity of viral proteins and antibody binding titres were maintained after doses of 3.6 Mrad[1] or 5 Mrad [3]. Irradiated specimens were tested for infectivity by inoculation onto Vero E6 cells, and examined for cytopathogenic effects for 10 days, followed by blind passage of the cells and testing for the growth of SARS-CoV by PCR[4].

Cryo-Electron microscopy. Virus samples (4μl) were applied to glow-discharged holey carbon films supported on 400-mesh copper grids. After blotting immediately for 2-5 seconds with filter paper, grids were plunged into liquid ethane cooled by liquid nitrogen, using a custom built gravity-operated freezing device. Specimens were transferred to a Tecnai 20 G2 transmission electron microscope (FEI) operated at 200kV, equipped with a Gatan 626.DH low-temperature specimen holder. Observations were made at temperatures of ~ -185°C and images recorded at 29,000X magnification on Kodak SO -163 electron image film at a dose of 10-20 electrons/Å^2 with an exposure of 1-2 seconds. Film was developed in Kodak D19 for 12 minutes at room temperature. Immuno-gold

stained samples were imaged at room temperature in the Tecnai 20, and digital images were collected using either a Gatan MSC or AMT Advantage XR-12 digital cameras.

Image processing. The exact magnification in the microscope was determined to be 29,968X using a calibration grid (Pelco International, Redding, CA). Images of SARS-CoV were digitised on a Nikon super coolscan 8000 ED scanner at a pixel size of 2.125Å. Processing was carried out using the EMAN and SPIDER/WEB image processing program packages[5,6] on SGI Fuel and Tezro workstations (SGI, Mountainview, CA). Images were corrected for contrast transfer function (ctf) using the "ctfit" function in the EMAN software package[7], which estimates defocus and corrects for ctf by phase-flipping. Images of the entire virus (n=140) were initially selected which consisted of population of spherical viruses, which represented the majority of the frozen-hydrated SARS-CoV virions. These images were analysed using the "cenalignint" function of EMAN, based on this data the average lipid bilayer envelope diameter was determined to be 865±44 Å. This diameter was then applied in the subsequent procedure to circularly average the envelope. Data sets composed of the nucleocapsid-centred images were grouped into two data sets recorded at approximately 8μ defocus (n=582) and 5.5μ defocus (n=637), as determined in the contrast transfer function correction. The images were analysed using the SPIDER software package using a multireference 2D alignment. This process included a step in which the reference images were circularly averaged as part of the pre-treatment procedure based on the average virus envelope diameter which was previously determined. The data sets composed of images centred on spikes (n= 8386; recorded at 3.3-12.7μ defocus) were processed using the projection matching approach[8] which incorporated three-fold symmetry into the search function and reconstruction procedure. The population of spike images was composed of both side view projections as well as end-on projections, with the latter being easily identified in the images with greater defocus values. These high-defocus/end-on images were essential to eliminate the "missing cone" from the reconstruction. The resolution of the cryo-EM reconstruction was estimated by Fourier shell correlation to be 16Å using the

12σ criteria. The structures of the pre-fusion HR2 domain and the receptor binding domain were docked into the cryo-EM reconstruction using the "colores" function in the SITUS software package[9]. The receptor binding domain atomic coordinates were extracted from the 2AJF.pdb[10] structure, these coordinates were then docked into the cryo-EM reconstruction. The resultant docking generated two distinct locations for the RBD (distal and proximal to the end of the spike). Each solution was then tested to see where the ACE-2 component (which was not used in the docking procedure) would be located. Any solutions in which the ACE-2 would lie within the mass of the spike were eliminated from the population of possible fits. The remaining fits were then compared to each other based on correlation and this indicated that the distal population had the higher correlation. Based on this the distal fit was considered best fit for the receptor binding domain. The docking of the entire 2FXP.pdb[11] structure was accomplished in a similar fashion also using SITUS with the exception that two functional considerations of the pre-fusion core had to be met for a docking to be considered meaningful. These were; firstly that the end of the HR2 pre-fusion core adjacent to the transmembrane domain had to point towards the viral envelope in the cryo-EM reconstruction, and secondly the symmetry axis of the 2FXP.pdb structure must lie on the same axis on the cryo-EM reconstruction.

Structure Visualisation. Three-dimensional cryo-EM reconstructions, and the atomic resolution structures 2AJF.pdb and 2FXP.pdb and were visualised using UCSF Chimera (Computer Graphics Laboratory, University of California, San Francisco, supported by NIH P41 RR-01081)[12]. Radial density shaded surface colours were produced using the Chimera extension available with EMAN[13]. The 1HA0.pdb[14] atomic structure was converted to an electron density map using EMAN. The spike component of the spike-viral envelope reconstruction was segmented from the entire reconstruction using the "floodfill" algorithm in the SITUS software package[15].

Immuno-Electron microscopy

Formvar-carbon coated 400-mesh nickel grids were floated on drops of purified SARS-

CoV (40µl) for 1 minute. All incubations were carried out at 20 °C. Grids were then washed in PBS, followed by a 10-minute block in PBS-G-BSA (PBS pH7.2, 0.2% glycine, 2% BSA). After washing in PBS-G (PBS pH7.2, 0.2% glycine), grids were then incubated in primary antibody diluted in PBS-G-BSA or convalescent patient serum diluted in PBS, followed by washes in PBS-G and then incubated in secondary antibody (conjugated to 5 or 10 nm gold), followed again by washing in PBS-G. Grids were fixed (1% paraformaldehyde, 1% glutaraldehyde in PBS), washed in deionised water and negatively stained in either 2% uranyl acetate or 2% methylamine tungstate (Nanoprobes, Yaphank, NY.). In the case of nucleocapsid labelling, specimens were permeabilised by pre-treatment with 0.05% NP-40 for in PBS on ice. In all experiments negative controls were run which included omission of the primary antibody to test for non-specific binding.

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